

PROTEIN TRANSPORT IN MOUSE KIDNEY UTILIZING TYROSINASE AS AN ULTRASTRUCTURAL TRACER*

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Physiological studies on renal clearance (1-5) have shown that molecules larger than about 68,000 mol wt are effectively restricted from entering the urinary space, while those molecules under 68,000 may be found in the glomerular filtrate. The degree of resistance encountered by a molecule in passing through the glomerulus is roughly proportional to its molecular weight. A portion of those exogenous proteins that do enter the urinary space are reabsorbed by the tubule cells. Ultrastructural studies employing either electron-opaque molecules (6-11) or enzymes capable of producing electron-opaque reaction products (12-17) have attempted to elucidate the morphological basis of glomerular filtration and protein uptake.

These studies have indicated that filtration barriers exist at two levels within the glomerulus, and that protein present in the glomerular filtrate is at least partially reabsorbed by the proximal tubule cells. However, a number of the available ultrastructural tracers are blocked at the glomerulus, and except for horseradish peroxidase (12) the lower molecular weight tracers have been difficult to use for the investigation of both glomerular filtration and protein reabsorption either because they are not very electron opaque, or they are rapidly excreted by the kidney.

In the present study mushroom tyrosinase subunits (mol wt 34,500) were employed as an ultrastructural tracer by taking advantage of the fact that oxidized dihydroxyphenylalanine (DOPA)¹ is electron opaque. Using this tracer it was possible to examine both glomerular filtration and subsequent protein uptake with a new, relatively low molecular weight tracer.

Materials and Methods

A solution of tyrosinase (Sigma Chemical Co., St. Louis, Mo.) was prepared by dissolving 5-30 mg in 0.1-0.5 ml physiological saline. The resulting solution was then injected into the tail veins of 25 adult male and female CFW mice (Carworth Laboratory Animals, New City, N.Y.). All concentrations of tyrosinase tested gave satisfactory results, but 0.3 ml of saline containing 15 mg of tyrosinase injected over a period of 60-90 sec produced the optimum results. At time intervals ranging from 45 sec to 30 min after completion of the injection, the mice were killed by cervical dislocation and the kidneys were removed.

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¹ *Abbreviation used in this paper:* DOPA, dihydroxyphenylalanine.

Preparation for Light Microscopy.—Thin slices (1–2 mm) of kidney were fixed either overnight in Formol-calcium (18) at 4°C or for 3 hr at 4°C in 2% glutaraldehyde containing 1% hydrogen peroxide (19). After fixation in glutaraldehyde the sections were rinsed in 0.2 M cacodylate buffer (pH 7.4) and left overnight at 4°C in buffer. 10- μ frozen sections were prepared on a Sartorius freezing microtome, collected briefly in distilled water, and then incubated as described below. After incubation, the sections were rinsed in distilled water and mounted in glycerin jelly on glass slides.

Preparation for Electron Microscopy.—The cortex of the kidney was cut into thin (1 \times 10 mm) strips. The strips were fixed for 4 hr at 4°C in 2% glutaraldehyde containing 1% hydrogen peroxide (19) in 0.2 M cacodylate buffer (pH 7.4). After fixation, the strips were rinsed and left overnight in buffer at 4°C. The strips were then chopped into 40- μ sections with a Smith and Farquhar tissue sectioner (Sorvall TC-2, Ivan Sorvall, Inc., Norwalk, Conn.). The sections were collected in buffer and incubated as described below. After incubation, the sections were refixed in 1% osmium tetroxide in 0.1 M phosphate buffer, dehydrated through a graded series of alcohols and propylene oxide, and embedded in Epon 812. Semithin, 1 μ sections were cut from the embedded material, and examined by light microscopy in order to evaluate the reaction. Thin sections were then cut with a diamond knife from suitable areas. The sections were mounted on bare copper grids. Some of the sections were lightly stained with lead citrate (20). All the sections were stabilized with carbon (21) and examined in a Siemens Elmiskop 101 (Siemens Corp., Medical Industrial Div., Iselin, N.J.).

Incubation Medium.—The DOPA-containing medium employed in this study was modified after that of Laidlaw and Blackberg (22). Fixed tissue sections were first soaked for 16–18 hr at 4°C in a medium containing 2 mg DOPA (Sigma) per ml of 0.2 M cacodylate buffer at pH 7.4. They were then incubated at 37°C for 4–5 hr in medium containing 4 mg DOPA per ml of buffer at pH 7.4. Occasionally at this pH, the DOPA underwent considerable autooxidation yielding a black solution; this did not seem to interfere with the tyrosinase reaction. However, at pH 8–9, autooxidation of the DOPA proceeded quite rapidly and considerable nonspecific adsorption occurred. Below pH 6.0 the reaction was almost entirely inhibited.

Controls.—Sections of kidney cortex from uninjected animals or from animals injected with saline were incubated and prepared for light and electron microscopy as described above. Sections from injected animals were also incubated in substrate-free medium.

Molecular Weight Determination.—The molecular weight of the Sigma tyrosinase was determined by dialysis and ultrafiltration. A tyrosinase solution was dialyzed for 24 hr in the cold in dialysis tubing (Union Carbide Corp., New York) which prevents passage of molecules larger than 15,000 mol wt. The tyrosinase solution was also subjected to ultrafiltration using a Diaflo Ultrafilter PM30 (Amicon Corp., Lexington, Mass.) that effectively restricts proteins with a mol wt of 30,000 \pm 5,000. Tyrosinase activity was assayed by a modification of the method recommended by Worthington Biochemical Corp., Freehold, N.J.

RESULTS

Light Microscopy.—Sections of the kidney cortex 45 sec after injection of tyrosinase (Fig. 1) show staining primarily within the glomerulus and peritubular capillaries. There is an indication of tyrosinase activity in the capillary lumen of the glomerulus and in the urinary spaces. The basement membrane of the glomerulus is darkly stained. The red blood cells present are also reactive. In contrast, the cortex of uninjected and saline-injected animals is unstained except for the red blood cells. Sections incubated in medium lacking substrate are also negative.

30 min after completion of the injection (Fig. 2) activity is demonstrable in

absorption droplets in the apical region of the proximal tubule cells. The uninjected and saline-injected animals showed no such staining when incubated in the DOPA-containing medium.

Electron Microscopy.—Almost immediately after injection tyrosinase reaction product can be observed both in the glomerulus and in areas of the brush border of the proximal tubules. The red blood cells are also reactive after incubation in the DOPA medium. In the glomerulus, accumulations of tyrosinase

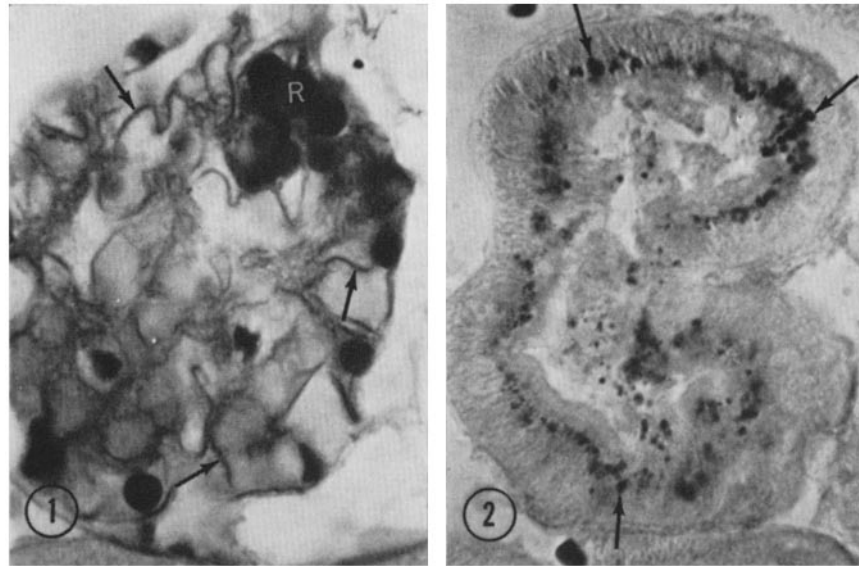


FIG. 1. Semithin section of glomerulus 45 sec after injection of 15 mg tyrosinase. The basement membrane (arrows) and red blood cells (*R*) are darkly stained. $\times 800$.

FIG. 2. 2% glutaraldehyde and 1% hydrogen peroxide-fixed frozen section of proximal tubule 30 min after injection of 5 mg tyrosinase. Tyrosinase reaction product is localized in absorption droplets (arrows) in the apical region of the cells. $\times 1,000$.

reaction product are visible in the capillary lumen (Fig. 3), endothelial fenestrae, and basement membrane (Fig. 4). Tyrosinase reaction product is present throughout the basement membrane with increased accumulations in both subendothelial and subepithelial layers (Fig. 5). In the subepithelial layer, a line of increased opacity is visible under the podocytes and in the slit pores extending to the level of the slit diaphragm. Some reaction product is present in the pores and in the urinary space (Fig. 7). The cell coat of the podocytes lining the urinary space also shows reaction product. In contrast, the podocytes from controls show no such accumulation, nor is there any indication of reaction product in the basement membrane, capillary lumen, or urinary space (Fig. 6).

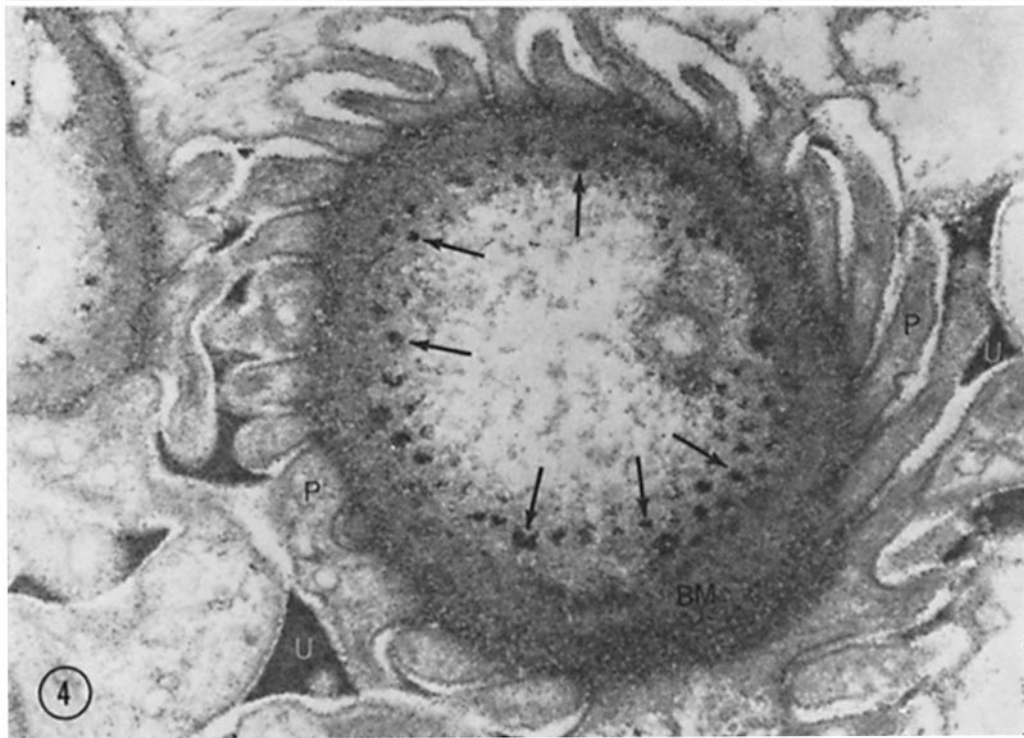
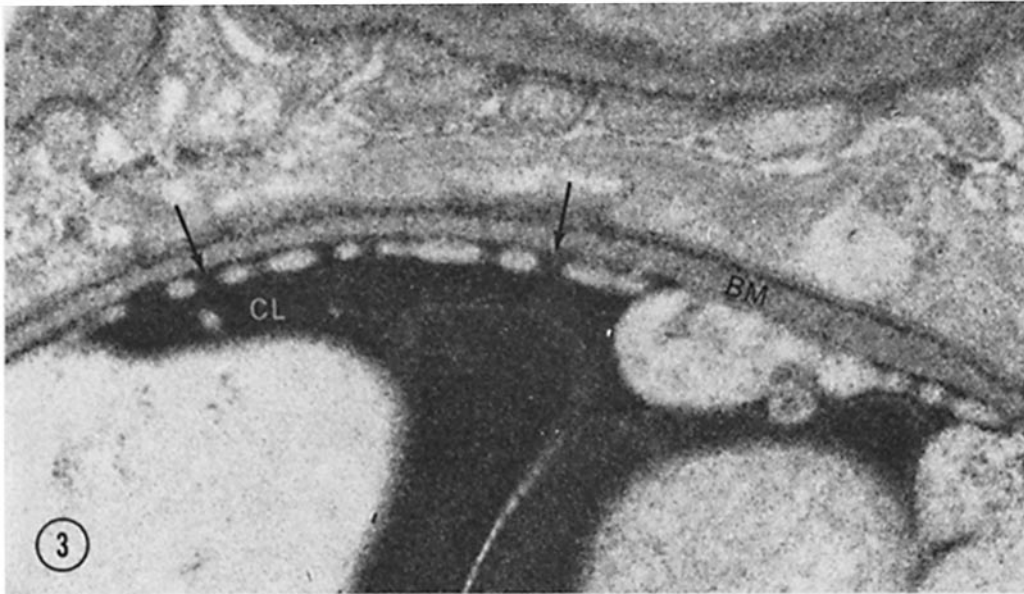


FIG. 3. Glomerulus 45 sec after injection of 15 mg tyrosinase. Tyrosinase reaction product is located in the capillary lumen (*CL*) within endothelial fenestrae (arrows) and in the basement membrane (*BM*). $\times 40,000$.

FIG. 4. Glomerulus 45 sec after injection of 15 mg tyrosinase. Tangential section of a capillary loop. Tyrosinase reaction product is visualized in the endothelial fenestrae (arrows) and the basement membrane (*BM*). Some reaction product is already found in the urinary space (*U*) and adjacent to the podocytes (*P*). $\times 14,200$.

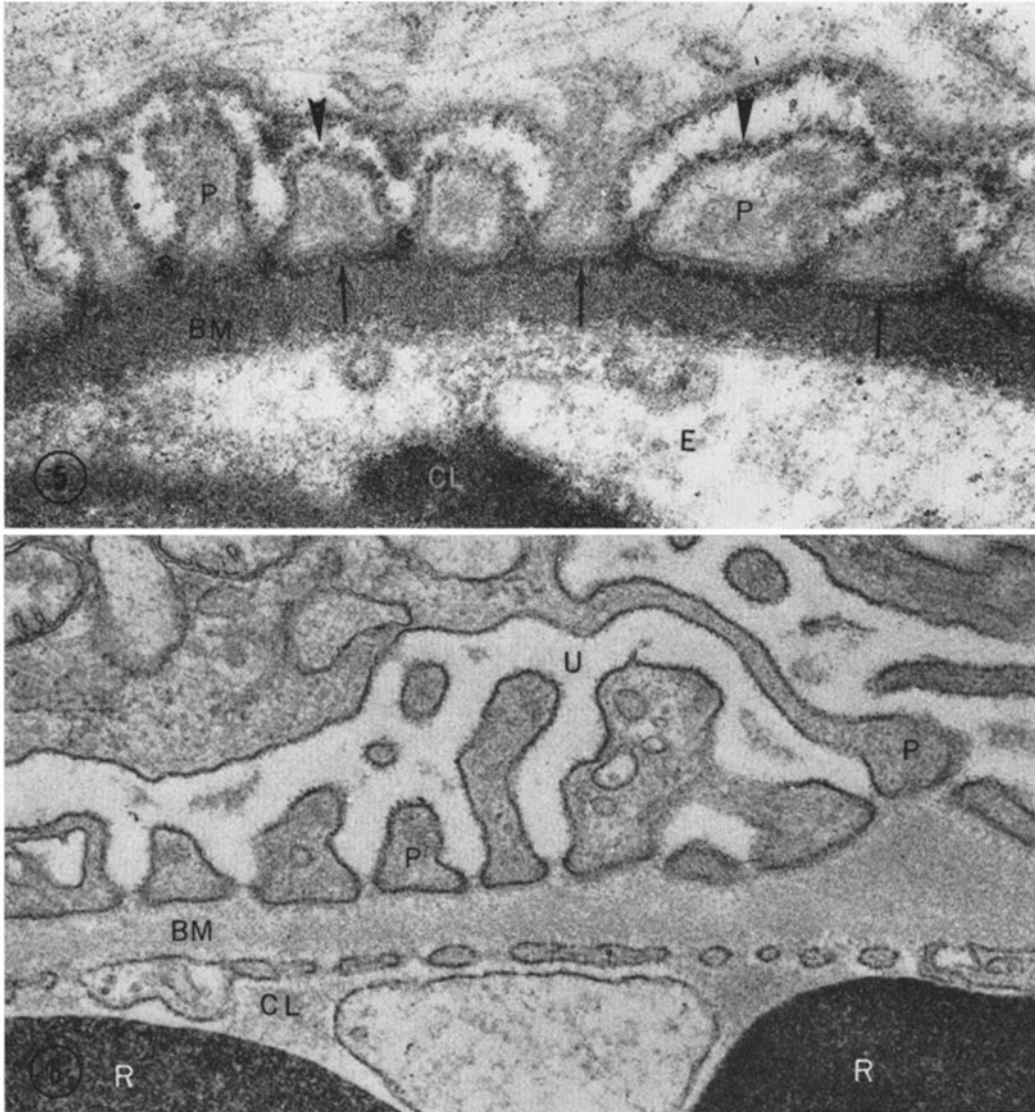


FIG. 5. Glomerulus 45 sec after injection of 15 mg tyrosinase. Tyrosinase reaction product is localized in the capillary lumen (CL) beneath the endothelium (E). The entire basement membrane (BM) is more opaque due to reaction product. The subepithelial layer (arrows) shows an increased accumulation of tyrosinase reaction product under the podocytes (P) and in the slit pores (S). The cell coat of the podocytes is also reactive (arrowheads). $\times 54,000$.

FIG. 6. Glomerulus from a saline-injected mouse, incubated in DOPA-containing medium. Although the red blood cells (R) show reaction product none is seen in the capillary lumen (CL), in the basement membrane (BM), on the surface of podocytes (P), or in the urinary space (U). $\times 51,000$.



FIG. 7. Glomerulus 45 sec after injection of 15 mg tyrosinase. Reaction product may be seen in the entire basement membrane (*BM*) and in increased accumulations in the subepithelial layer of the basement membrane (arrows). It is also present in the urinary space (*U*) and on the surface of the podocytes (*P*). $\times 19,000$.

At the earliest times after injection, the reaction product in the proximal tubule is always less dense than that in the glomerulus. There is a small amount of tyrosinase reaction product between the microvilli and in the terminal web of the brush border (Fig. 8), but there is no noticeable uptake into the tubule cells.

By 20 min after injection of tyrosinase, little or no reaction product is visible in the glomeruli except for vesicles within mesangial cells. In the proximal

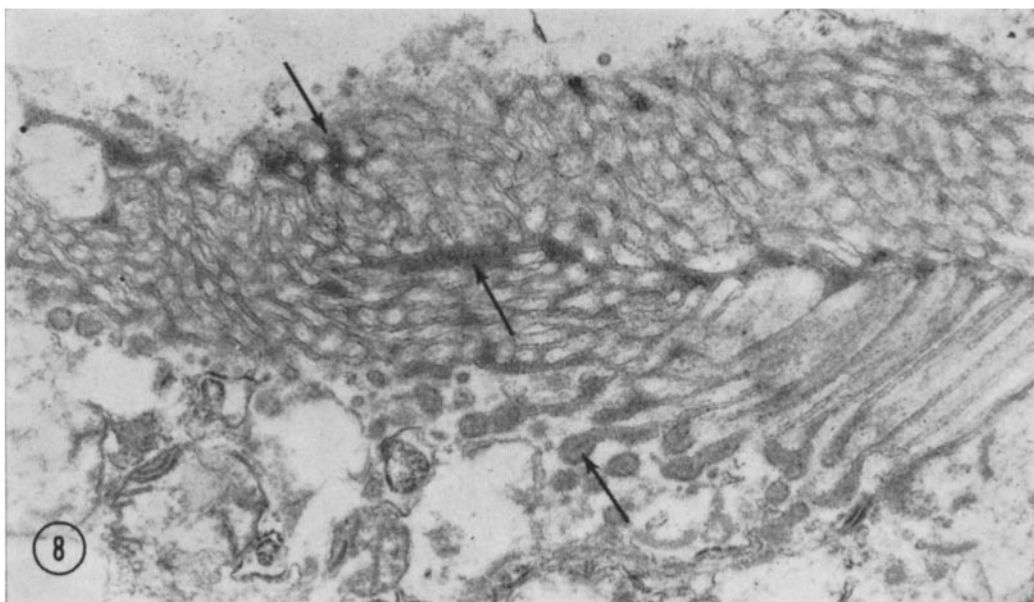


FIG. 8. Apical region of proximal tubule cell 45 sec after injection of 15 mg tyrosinase. Reaction product is visible between the microvilli of the brush border and in apical invaginations (arrows). $\times 20,000$.

tubules (Fig. 9), the tyrosinase reaction product is present in absorption droplets, located at the base of the brush border. Reaction product can also often be visualized in the peritubular capillaries and occasionally it is seen within the basal infoldings of the proximal tubule cells (Fig. 10). There is also reaction product localized in the basement membrane (Fig. 10). The distribution of the reaction product is similar to that seen at earlier times in the glomerular basement membrane (Fig. 5).

MOLECULAR WEIGHT DETERMINATION

After dialysis, the majority of the tyrosinase activity remained inside the tubing. However, after filtration, approximately two-thirds of the activity was

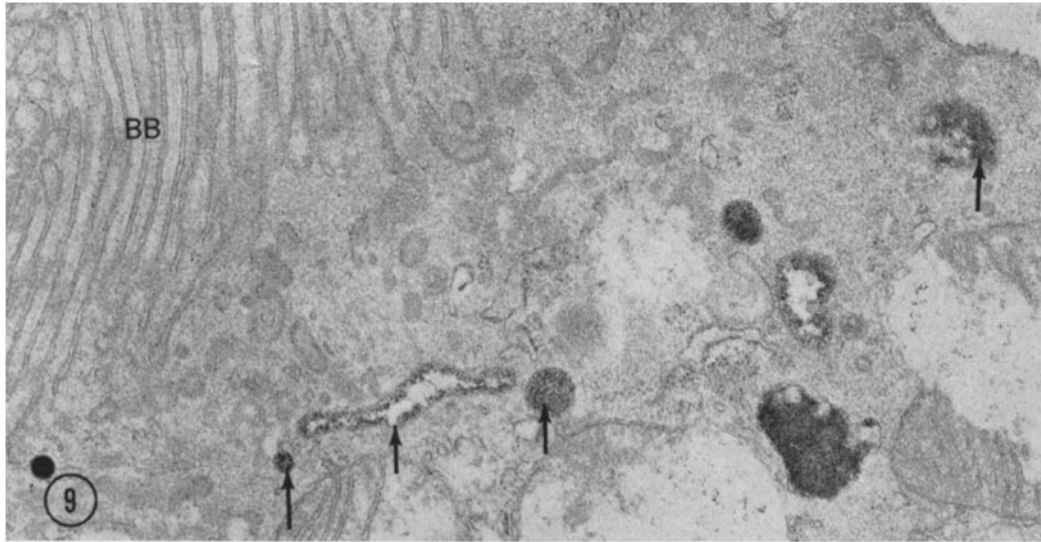


FIG. 9. Apical region of proximal tubule cell 20 min after injection of 15 mg tyrosinase. The reaction product is located in absorption droplets (arrows) adjacent to the brush border (*BB*). $\times 25,000$.

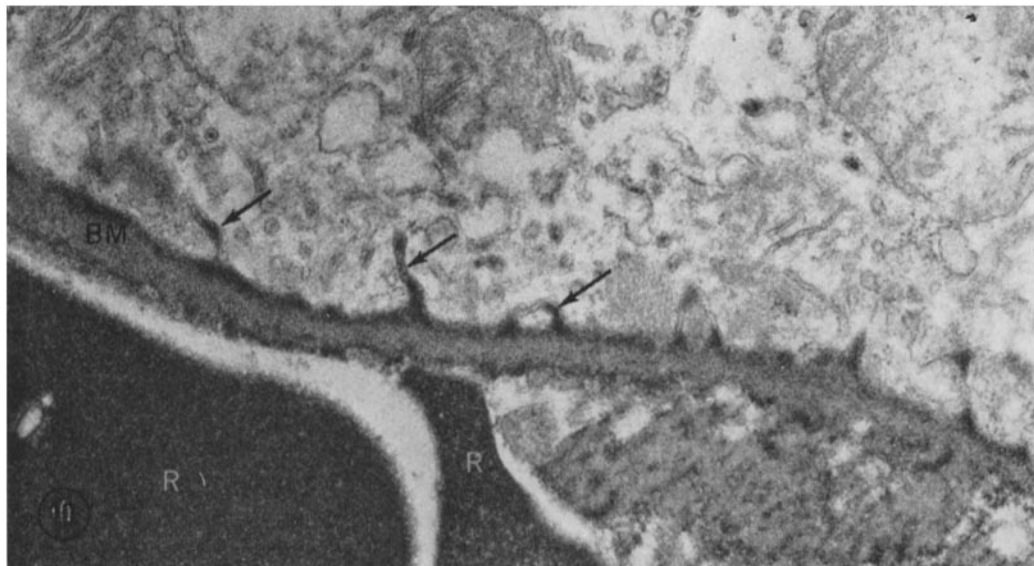


FIG. 10. Basal region of proximal tubule cell 30 min after injection of 15 mg tyrosinase. Reaction product is visible in the red blood cells (*R*) of the peritubular capillary and in the basal infoldings (arrows) of the proximal tubule cells. The basement membrane (*BM*) shows an accumulation of reaction product similar to that seen previously in the glomerular basement membrane (Fig. 5). $\times 27,000$.

found in the filtrate. This analysis places the mol wt of the tyrosinase around $30,000 \pm 5,000$. This is in agreement with a mol wt of 34,500 given for the subunits of mushroom tyrosinase, prepared by treatment of tyrosinase with sodium dodecyl sulfate (23). It is also in agreement with a mol wt of 34,500 given for the commercial preparation of tyrosinase (Sigma) prepared according to the method of Kertesz and Zito (24). Therefore, it is concluded that tyrosinase as supplied by Sigma is in the form of active subunits of 34,500 mol wt rather than as the native molecule of 116,000 mol wt (25).

DISCUSSION

The results of this study indicate that intravenously injected tyrosinase subunits, (mol wt 34,500) are transported by the kidney, in a manner similar to that of horseradish peroxidase (mol wt 40,000) (12, 13). Tyrosinase appears to pass rapidly from the capillary lumen, through the endothelial fenestrae, and into the basement membrane where it is present in greater concentration in both subendothelial and subepithelial layers. Some tyrosinase is also taken up by mesangial cells in the glomerulus. Part of the tyrosinase passes through the slit pores between the foot processes and enters the urinary space where some is adsorbed to the cell coat of the podocytes. At least a portion of the tyrosinase remaining in the glomerular filtrate is taken up into absorption droplets by the proximal tubule cells. Occasionally, reaction product is found in the basal infoldings of these cells.

Previous studies on glomerular filtration employing ultrastructural tracers have indicated that passage through the glomerulus is largely a function of molecular size. Table I lists the most common ultrastructural tracers employed in the study of glomerular filtration. Tyrosinase does not seem to be greatly impeded by any filtration barriers since appreciable reaction product is present within the urinary space as early as 45 sec after injection. However, there are accumulations of tyrosinase, as judged by increased opacity of reaction product, primarily in the subepithelial layer, and to a lesser extent, subendothelial layer of the glomerular basement membrane. Earlier work has suggested the presence of filtration barriers at two levels within the glomerulus. Large molecular weight molecules (Table I) such as Thorotrast (26), ferritin (8, 27, 28), and saccharated iron oxide (29) are able to pass through the endothelial fenestrae and enter the subendothelial layer of the basement membrane, but their movement across the membrane is markedly restricted. Catalase (17) with a mol wt of 240,000 exhibited a gradient across the basement membrane with the highest opacity occurring in the subendothelial layer of the membrane, indicating a blockage primarily at the lamina densa. In contrast, an intermediate molecular weight tracer (Table I), such as myeloperoxidase, (mol wt 170,000) (12), was able to traverse the entire basement membrane, but was blocked at the level of the epithelial slit pores with an accumulation of reaction product in the subepithelial layer. Lactoperoxidase (mol wt 82,000) (14) behaved simi-

larly to myeloperoxidase, but more lactoperoxidase was present in the glomerular filtrate. Although neither peroxidase nor tyrosinase is prevented from entering the urinary space, both show accumulations of reaction product in the subendothelial and subepithelial layers of the basement membrane. It has been suggested (28) that accumulation of ultrastructural tracers in the inner and outer layers of the basement membrane is due simply to a less compact molecular arrangement of these layers in comparison to the dense inner layer. The less compact layers are thus able to accommodate more tracer within their molecular framework. The lamina densa acts as a coarse filter preventing

TABLE I
Ultrastructural Tracers Used in the Study of Glomerular Filtration

Tracer	Mol wt	Reference*
High molecular weight tracers (blocked at lamina densa)		
Thorotrast	—	Latta et al., 1960 (26)
Ferritin	500,000	Farquhar et al., 1961 (8)
Saccharated iron oxide	—	Deodar et al., 1964 (29)
Intermediate molecular weight tracers (blocked at epithelial slit pore)		
Beef liver catalase	240,000	Venkatachalam et al., 1970 (17)
Human myeloperoxidase	170,000	Graham and Karnovsky, 1966 (13)
Bovine lactoperoxidase	82,000	Graham and Kellermeier, 1968 (14)
Low molecular weight tracers (enter urinary space)		
Ox hemoglobin	68,000	Miller and Palade, 1964 (9)
Horseradish peroxidase	40,000	Graham and Karnovsky, 1966 (12, 13)
Equine cytochrome <i>c</i>	12,000	Karnovsky and Rice, 1969 (15)

* Numbers in parentheses refer to reference numbers.

passage of molecules with a diameter larger than 100 Å (26). This could account for an accumulation of enzyme in the subendothelial layer. If more protein were present in the basement membrane than the lamina densa could accommodate, it might accumulate in the subendothelial layer. Similarly, the slit pores also act as filtration barriers, and enzyme may be held temporarily in the subepithelial layer before it is able to enter the urinary space. However, Jones (30) and Groniowski et al. (31) have suggested that the inner and outer layers of the basement membrane may contain small amounts of sialic acid as well as other glycoproteins, which may act to trap proteins. When sections of kidney were exposed to colloidal iron, the subendothelial and subepithelial layers of the basement membrane showed a light accumulation. Whether this

is real or artifactual is still open to question. Jones (30) has shown that the iron could be removed by hyaluronidase but not by neuraminidase. However, Groniowski et al. (31) report that the colloidal iron could be removed by neuraminidase indicating that this localization may be due to sialic acid. The concentrations of enzyme reaction products in the inner and outer layers of the basement membrane therefore might be explained not only by differences in structure of these layers, but also by the presence of an acid mucosubstance known to have protein-concentrating ability (30).

The acid mucosubstance coat of the podocytes may also play a role in removing proteins from the glomerular filtrate (30, 31). The reaction products of both tyrosinase and peroxidase (12) were visualized on the cell coats of the podocytes, suggesting adsorption of enzyme. The adsorption of tyrosinase to the cell coat could reduce its concentration, and may partially explain the lowered reactivity of the glomerular filtrate in the lumen of the proximal tubules. Once the tyrosinase was concentrated into adsorption droplets, an opaque reaction product was again observed.

The fate of the adsorbed protein is not clear. It has been demonstrated that ferritin may be taken up into glomerular epithelial cells either from the urinary space (32) or from the basement membrane (8). However, since no reaction product was found in the epithelial cells after injection, this does not appear to be a major pathway in this case.

The tyrosinase reaction product which is seen in the mesangial cells 30 min after injection probably reflects the uptake, from the basement membrane and the mesangial matrix of protein which did not enter the urinary space. When ferritin (27), Thorotrast (26), catalase (17), myeloperoxidase, and peroxidase (12), are injected intravenously, they are first seen in the basement membrane, and spongy areas around the mesangial cells, and then within the mesangial cells, where the protein is apparently digested.

The presence of tyrosinase reaction product within the basal infoldings of the proximal tubule cells may also be due to adsorption onto the cell coat of these cells. Staining with ruthenium red (31) has shown the presence of a glycoprotein coat on the membranes of the basal infoldings. Although there is no indication of any uptake of tyrosinase into the tubule cells through the basal infoldings, vital dyes have been shown to enter the cells through these channels (19), and it is possible that small amounts of protein or peptides may enter the cells through the basal cytoplasm.

In the proximal tubule cells tyrosinase appears to be taken up in a manner similar to that already demonstrated for other proteins. Tyrosinase reaction product was found between the microvilli of the brush border and in the terminal web; subsequently it could be visualized in absorption droplets in the apical cytoplasm.

The results of this study show that tyrosinase subunits behave in the kidney as do other proteins of similar molecular weight. It is suggested that tyrosinase subunits may be useful as an additional tracer for the study of protein transport.

SUMMARY

The morphological basis of glomerular filtration and protein reabsorption in mouse kidney was examined by using mushroom tyrosinase subunits (mol wt 34,500), as an ultrastructural tracer. Almost immediately after injection tyrosinase reaction product was visualized in the glomerulus, and within the capillary lumen extending into the endothelial fenestrae. The entire basement membrane showed accumulations of tyrosinase in the subendothelial and subepithelial layers. The urinary space contained considerable amounts of reaction product, some of which was adsorbed to the cell coat of the podocytes. Reaction product could also be seen in the brush border region of the proximal tubule cells. By 30 min after injection, no tyrosinase reaction product was demonstrable in the glomerulus except for dense vesicles in mesangial cells. Most of the reaction product was localized in absorption droplets in the apical cytoplasm of proximal tubule cells. Occasionally, some tyrosinase reaction product was present within the basal infoldings of these cells. The behavior of tyrosinase in the mouse kidney is in accordance with that of other low molecular weight tracers. The pattern of localization within the basement membrane provides additional support for the presence of two filtration barriers in the glomerulus. The adherence of tyrosinase to the cell coat of the glomerular epithelial cells suggests that this may be an additional mechanism whereby protein is removed from the glomerular filtrate. Tyrosinase subunits may prove to be a useful new tracer for the study of protein transport.

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